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POLYMORPHISM I : LINKAGE OF ASTHMA TO A LOCUS ON CHROMOSOME 2

This invention is concerned with methods for the diagnosis of asthma and with materials and methods relating thereto.

Asthma is a disease which is becoming more prevalent and is the most common disease of childhood (1). Most asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so that factors in addition to atopy are necessary to induce the disease (2,3). Asthma is strongly familial, and is due to the interaction between genetic and environmental factors. The genetic factors are thought to be variants of normal genes ("polymorphisms") which alter their function to predispose to asthma.

Asthma may be identified by recurrent wheeze and intermittent air flow limitation. An asthmatic tendency may be quantified by the measurement of bronchial hyper-responsiveness in which an individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in air flow (PD20) or the slope of the curve between the initial air flow measurement and the last dose given (slope).

In the atopic response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE-coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a series of cellular events leading to the destabilisation of the cell membrane

and release of inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE.

Genetic factors underlying a disease may be identified through localisation to particular chromosomal regions by genetic linkage. Genetic linkage is established by the study of families. It relies on matching the inheritance of disease with genetic polymorphisms of known localisation (known as "genetic markers"). In a complex disease such as asthma, genetic linkage will typically localise genes to within 10 - 20 Megabases (Mb) of DNA. A region of this size may contain 350 - 700 genes, and will be too large to permit immediate identification of the disease-causing gene.

Closer localisation of disease-causing genes may be accomplished by the detection of associations between particular alleles and the disease phenotype. Over short segments of DNA, distinctive alleles of the individual polymorphisms will show non-random association with alleles of neighbouring polymorphisms. This phenomenon, known as "linkage disequilibrium" occurs over 50-500 Kilobases (Kb) of DNA. Linkage disequilibrium may be detected by the study of individuals as well as by the study of families.

Disease-causing alleles will be in linkage disequilibrium with non-functional polymorphisms from the same chromosomal segment. It is therefore possible to detect allelic association with disease from particular chromosomal segments, without identifying the exact polymorphism and gene underlying the disease state.

The detection of allelic association may therefore give information as to disease susceptibility in a particular individual. Furthermore,

allelic association is indicative of a disease-causing gene being present within 500 Kb of DNA in either direction from the allele (i.e. 1 Mb in total). Such a region may contain only 30 genes, within which the identification of the disease-causing gene is possible.

The presence of linkage disequilibrium also means that other polymorphisms may be anticipated to associate with disease, and that these additional polymorphisms will also be diagnostic of disease susceptibility in particular individuals.

Genetic associations with atopy have been demonstrated. WO 95/05481 discloses that variants of the gene encoding the β-subunit of the high-affinity receptor for IgE (FcεRIβ) are associated with atopy. It teaches a method for diagnosing atopy which is based upon the demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding FcεRIβ, located near the commencement of exon 6 of the FcεRIβ gene on chromosome 11. A further variant has also been found in which the unusual variant sequence is in the coding sequence for the C-terminal cytoplasmic tail of FcεRIβ (4).

Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (5). We have previously shown that polymorphisms within the TNF gene are associated with an increased risk of asthma (6).

The known polymorphisms do not account for all of the genetic factors which predispose to asthma. In particular, asthma is not necessarily an atopic disease. Identification of further genetic polymorphisms linked to asthma will allow the identification of children at risk of asthma before the disease has developed (for example immediately after birth), with the potential for prevention of disease. The presence of particular polymorphisms may predict the clinical course of disease (e.g. severe as opposed to mild) or the

response to particular treatments. This diagnostic information will be of use to the health care, pharmaceutical and insurance industries.

It has now been discovered that a genetic polymorphism known as D2S308*3 near the IL1 gene cluster on chromosome 2 is associated with asthma and can be used as a diagnostic tool.

Interleukin 1 (IL1) is another pro-inflammatory cytokine which is increased in asthmatic airways (5). IL1 is part of a gene family, localised on the long arm of chromosome 2 (7). Not all members of the gene family have been identified. The members of the family may share similar functions.

The invention therefore provides a method for diagnosing an individual as being asthmatic, or as having a predisposition to asthma, which method comprises demonstrating in the individual the presence or absence of an allele which is associated with asthma, wherein the allele is situated at a locus in a region of chromosome 2 of up to 1 megabase in length, which region contains the locus D2S308.

The 1 Mb region of chromosome 2 in which the D2S308 locus is situated flanks the D2S308 locus. Thus, the specific allele D2S308*3, or other unusual polymorphisms in the region which are associated with asthma, may be the subject of identification in the method according to the invention. Equally, two or more such alleles may be the subject of identification.

Current diagnostic methods involving detection at the nucleic acid level normally comprise the steps of:

(i) obtaining a suitable tissue sample from the individual;

(ii) preparing from the tissue sample a nucleic acid sample;

(iii) analysing the nucleic acid sample for the presence or absence of the relevant nucleic acid sequence, such as a specific allele.

Preferably, an amplification step is performed prior to the analysis, such that the locus at which the allele is situated is amplified. A preferred amplification technique is the PCR, although any suitable method of nucleic acid amplification may be employed.

In further aspects, the invention provides a pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 2 of up to 1 megabase in length, which region contains the locus D2S308; and an assay kit comprising the pair of oligonucleotide primers.

The specific allele for identification may take the form of microsatellite repeats, which are nucleotide sequences containing short, repeated nucleotide motifs, usually a dinucleotide or a trinucleotide motif. A pair of primers which hybridize under suitably stringent conditions, to sequences at a position on either side of the microsatellite repeats, may be used to amplify the microsatellite repeats by PCR. Differences in the number of repeats are recognised by size differences in the PCR products. An allele which has a specified number of repeats and therefore a known size can thus be identified. D2S308*3 is one such allele.

The primers employed in the method comprise nucleic acid sequences which are complementary to, or substantially complementary to unique sequences either side of the microsatellite repeats, such that only the relevant polymorphic region of the genome is amplified. The conditions under which the amplification is performed are gauged such that specific hybridization of the primers to the flanking sequences occurs and non-specific hybridization is avoided. The hybridization conditions are suitably stringent for that purpose. Standard techniques can be used to identify an appropriate set of reaction conditions.

Typically, the PCR products are detected by means of a detectable label attached to one of the PCR primers. Alternatively another form of labeling may be used such as a labeled sequence specific probe which hybridizes to the amplified sequences. The label may be a fluorescent or other label. The PCR products are subjected to size determination, typically involving size-separation for example by gel electrophoresis, and the presence or absence of the allele of interest is determined.

It will be evident that the invention is not limited with regard to the manner in which the presence or absence of the allele of interest is determined. The labeling, detection, separation or any other aspect of the method as described here may be replaced by other suitable known techniques and reagents.

The allele for identification may be an allele other than D2S308*3 which is in linkage disequilibrium with D2S308*3 and is associated with asthma. This includes alleles of both functional and non-functional polymorphisms. Functional polymorphisms include polymorphisms within genes, usually within coding sequences of genes. Non-functional polymorphisms are polymorphisms which do not themselves cause the disease.

This invention will now be further described in the Examples section which follows. The Examples are intended to be illustrative and do not limit the scope of the invention in any way.

EXAMPLES

Description of Laboratory Testing

Subjects

Three panels of subjects have been studied.

Panel A consisted of 80 nuclear families sub-selected from an

Australian population sample of 230 families (8). The panel contained a total of 203 offspring forming 172 sib-pairs. 12% of the children were asthmatic.

Panel B consisted of 77 nuclear and extended families recruited from asthma and allergy clinics in the United Kingdom (9). These families contained 215 offspring (268 sib-pairs) of which 56% were asthmatic.

Panel C consisted of 87 nuclear families recruited through a child attending an asthma clinic in the Oxford region. The families contained 216 offspring (148 sibling pairs), of whom 44% were asthmatic.

Phenotyping

The subjects were administered a modified British MRC respiratory questionnaire. "Asthma" was defined as a positive answer to the questions "Have you ever had an attack of asthma?" and "If yes, has this happened on more than one occasion?". "Wheeze" was defined as a positive answer to the question "Has your chest ever sounded wheezing or whistling?" and "If yes, has this happened on more than one occasion?". The total serum IgE was measured in all children. Skin tests to house dust mite and grass pollen were carried out.

Genotyping

The microsatellite markers D2S340, D2S160, IL1A, D2S308 and D2S121 were typed by semi-automated fluorescent methods, as described previously (8). These markers cover a distance of 12 centiMorgans (approximately 12 million base pairs of DNA), containing thc IL1 cluster of genes.

The polymerase chain reaction (PCR) primer sequences for the markers were as follows:

D2S308 5' GCC AGG TGG GTC TGG A (SEQ ID NO: 1)

UD2S308 5' TGG TTT TTT CAA GGG ATT TTC T (SEQ ID NO: 2)

D2S340 5' CTG GAA CCC CAG TAG C (SEQ ID NO: 3)
UD2S340 5' CTT TGC CCA GTT TTG C (SEQ ID NO: 4)

D2S160 5' TGT ACC TAA GCC CAC CCT TTA GAG C (SEQ ID NO: 5)
UD2S160 5' TGG CCT CCA GAA ACC TCC AA (SEQ ID NO: 6)

IL1A 5' GCC TAG TGA GTG TGG AAG ACA TTG (SEQ ID NO: 7)
UIL1A 5' CAG CAC TGG TTG GTC TTC ATC TTG (SEQ ID NO: 8)

D2S121 5' GCT GAT ATT CTG GTG GGA AA (SEQ ID NO: 9)
UD2S121 5' GGC AAG AGC AAA ACT CTGTC (SEQ ID NO: 10)

The polymerase chain reaction conditions were as follows: The reaction volumes were 10µl, containing 50ng of genomic DNA, 200mM dNTPs, I x NH4+ buffer, 50ng oligonucleotide primers (forward labelled fluorescently), 0.5 to 3.0mM $MgCl_2$ and 0.2U Taq polymerase. Cycling conditions were 1 min at 95°C, 1min at 55°C and 45s at 72°C; 28 cycles were used. PCRs were performed on an Hybaid Omnigene thermal cycler.

Electrophoresis and allele scoring were as follows: PCR products were mixed with a size standard (GS350 TAM) in loading buffer (80% (v/v) formamide, 20% (v/v) 50mM EDTA. 0.1% (w/v) blue dextran). Samples were denatured at 95°C for 4min immediately prior to loading onto a 6% polyacrylamide gel and were electrophoresed at 800v for 6h on an Applied Biosystems (ABI) 373 DNA sequencer. Allele sizes were assigned using the ABI GENESCAN and ABI GENOTYPER software.

Linkage and Association Analysis

Linkage was tested against asthma and the associated

phenotypes of wheeze, the skin test index, the total serum IgE and the residual IgE (the proportion of the total IgE not attributable to specific allergy)(8,10). Linkage was assessed by standard sib-pair methods as described (8).

Association was tested against the phenotype of asthma by the Transmission Disequilibrium Test.

Results

Genetic linkage to asthma-associated phenotypes was detected in Panel A and Panel B subjects as follows (genetic linkage to these phenotypes was not detected in Panel C subjects):

		Skin Test Index		Residual IgE		Wheeze	
	θ	Panel A	Panel B	Panel A	Panel B	Panel A	Panel B
D2S340	0.04	-	-	-	-	p<0.01	-
D2S160	0.02	-	p<0.005	p<0.01**	-	p<0.01	p<0.05
IL1A	0.05	-	-	-	-	p<0.0005	-
D2S308	0.01	-	-	-	-	p<0.005	-
D2S121		-	-	-	-	p<0.01	-

*recombination fraction: 0.01 approximates 1 million base pairs

**p<0.00001 in maternal meioses

Genetic linkage between the marker D2S160 and asthma was detected at p=0.09 in Panel A, p=0.01 in Panel B, and p=0.12 in Panel C. The combined significance of linkage was p=0.008.

These results indicate that a gene influencing asthma lies within the D2S340 - D2S121 interval. Based on the current linkage information, the precise localisation of the putative asthma gene within the interval is uncertain.

Association with D2S308 allele 3

Each of the markers was then tested for association with asthma phenotype. Association was seen in each panel for allele 3 of D2S308 (D2S308*3). This allele is 228 base pairs in size, using the primers described above. (Other primers can be designed and their amplification product size determined for D2S308*3, using known sequence in formation (11).) The results of TDT testing were as follows:

	Transmitted	Not Transmitted	p
Panel A	14	4	p=0.015
Panel B	55	29	p=0.003
Panel C	49	32	p=0.037
Combined	118	65	p=0.00009

The results indicate that D2S308*3, near the Interleukin 1 gene cluster on chromosome 2, shows a strong reproducible association with asthma in three diverse panels of subjects. It may therefore be inferred that a gene influencing asthma is present within 500 kilobases in either direction of D2S308.

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5